THE ACCUMULATION OF PHOSPHATE BY FERTILIZED SEA URCHIN EGGS [1]

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Radioactive phosphate enters fertilized sea urchin eggs far more rapidly than it enters the unfertilized eggs (Brooks and Chambers, 1948, 1954; Abelson, 1947; Lindberg, 1948; Whiteley, 1949). Investigations described in this paper demonstrate that the entry of P^{32} into the eggs represents an accumulation of phosphate within the eggs. In addition, the concentrations of P and P^{32} in the inorganic and organically bound phosphate fractions of the eggs have been measured, with the purpose of determining in which fractions the phosphate, accumulated by the fertilized eggs, is incorporated, and whether the process of accumulation is associated with alterations in the distribution of P within the eggs.

Materials and Methods

Eggs of the Pacific coast sea urchins *Strongylocentrotus purpuratus, S. franciscanus,* and *Lytechinus pictus* were prepared for use, and measurements of egg volume, and of P^{32} concentration in the eggs and suspension fluid carried out as described previously (Brooks and Chambers, 1954). The jelly was removed from the eggs by repeated washings in sea water. The sea water used in the experiments was filtered through fine mesh filter paper. The experiments were performed at $15 \pm 0.1°$ C. unless otherwise stated. The pH of the sea water in which the eggs were suspended was measured at intervals throughout the duration of the experiments, and varied between pH 8.0 to 8.2. Carrier-free P^{32}, as orthophosphate, was added to the egg suspensions in amounts which varied from 0.2 to 2 μc P^{32}/liter of suspension. The concentration of orthophosphate in the sea water was measured using the Deniges-Atkins method (Atkins, 1923) with corrections for reagent blank and salt error (Cooper, 1938).

Trichloroacetic acid extracts of unfertilized and fertilized eggs were prepared as described by Chambers and Mende (1953a). Measurements of the P and P^{32} content of the inorganic and easily hydrolyzable phosphate fractions of the trichloroacetic acid-soluble extracts were carried out using the isobutyl alcohol extraction method of Borbiro and Szent-Györgyi (1949). After measurement of the phosphomolybdate concentration in the isobutyl alcohol extracts, aliquots were pipetted into flat dishes, evaporated, and the P^{32} concentration measured using a Geiger-Müller counter. In all experiments described in this paper samples of the egg

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suspensions, as originally prepared, were kept for observation. If the suspension was of unfertilized eggs, these were inseminated at the completion of the experiment. Over 95 per cent of the eggs in these samples developed to normal swimming gastrulae.

Results

Removal of P and P^{32} from the suspension fluid, and uptake of P^{32} by the eggs in suspensions of sea urchin eggs

Suspensions of unfertilized and fertilized eggs were prepared in filtered sea water containing 2.5 to 5.6 ml. eggs/liter. Small quantities of orthophosphate and P^{32} were added to the suspensions. The initial concentration of orthophosphate in the suspension fluid varied between less than 4 μg to 78 μg P/liter, and the initial concentration of P^{32} from 0.23 to 0.28 μc/liter.

Unfertilized eggs. The results obtained using suspensions of unfertilized *S. purpuratus* eggs are shown in Table I. Measurements of P and P^{32} concentrations were begun two hours after the eggs had been removed from the ovaries. In Experiments 1, 2 and 3 the concentration of P in the external medium increased,

Table I

Concentration of P in the suspension fluid of a suspension of unfertilized S. purpuratus eggs. Experiments 1 to 5

Expt. No.	Ml. eggs/l. suspension	Time between P analyses in minutes	Initial P conc., μg/l. susp. fluid	Final P conc., μg/l. susp. fluid	μg P lost/ ml. eggs/min.	μg P entering/ ml. eggs/min.
1	5.6	97	38	55	0.032	0.0011
2	4.1	120	8	20	0.024	—
3	4.7	312	30	56	0.019	—
4	5.5	115	4	5	0.0	—
5	2.5	159	78	78	0.0	0.0026

while in Experiments 4 and 5 no appreciable change in the P concentration could be detected (Table I, columns 4, 5 and 6). There was no measurable change in concentration of P^{32} in the suspension fluid in any of the experiments. In Experiments 1 and 5 the uptake of P^{32} by the eggs was measured and the quantity of P entering one ml. eggs/minute calculated (Table I, column 7), as previously described by Brooks and Chambers (1954). The results reveal that P enters unfertilized eggs whether or not the eggs simultaneously lose P to the external medium. At the completion of the experiments the unfertilized eggs were inseminated, and they developed normally through the gastrula stage.

Fertilized eggs. The results obtained using suspensions of fertilized *S. purpuratus* and *S. franciscanus* eggs are shown in Tables II and III, and Figure 1. The eggs were inseminated two hours after removal from the ovaries, washed free of spermatozoa by gentle centrifugation, and suspended in sea water containing known concentrations of orthophosphate and P^{32}.

In Experiment 6 (Table II, Fig. 1) at 20.5 minutes after insemination 2.5 ml. of *S. purpuratus* eggs were suspended in a liter of sea water containing 78 μg P/liter. A prominent decrease in concentration of P and P^{32} in the medium occurred (Expt.

6, Table II, columns 2 and 4, and Fig. 1). The rate of uptake of P^{32} by the eggs (Table II, column 6) was identical to the rate of disappearance of P and of P^{32} from the medium (Table II, compare columns 3, 5 and 7). The initial lag in the disappearance of orthophosphate and of P^{32} from the medium (Fig. 1, Expt. 6, from 0 to 30 minutes) is due to the fact that the uptake of P by fertilized eggs does not reach a maximum until about one hour after insemination (Brooks and Chambers, 1954). Subsequently, orthophosphate is removed from the medium at a constant rate until the concentration falls to 15 to 20 μg P/liter (Fig. 1, Expt. 6). The rate of uptake then falls off sharply.

TABLE II

Concentration of P and P^{32} in the suspension fluid, and of P^{32} in the eggs in suspensions of fertilized eggs.

Experiments 6 and 7

Time after initial measurement in minutes	μg P/l. susp. fluid	Per cent initial P conc. in susp. fluid	CPM P^{32}/l. susp. fluid	Per cent initial P^{32} conc. in susp. fluid	CPM P^{32} in eggs/l. suspension	Per cent initial P^{32} conc. in eggs
Experiment 6. *S. purpuratus* eggs						
0.0*	78	100.0	61,000	100.0	0	0.0
17.5	75	96.0	56,600	92.6	4,780	7.8
60.5	48	61.5	36,780	60.2	24,900	40.8
91.5	22	28.2	16,960	27.8	42,920	70.5
129.5	6	7.7	6,600	10.8	54,480	90.0
213.0	<2	<3.0	3,200	5.2	58,140	95.5
387.5	<2	<3.0	2,360	3.8	—	—
Experiment 7. *S. franciscanus* eggs						
0.0†	53.5	100.0	76,000	100.0	0	0.0
31.0	40.0	74.8	62,000	81.6	15,200	20.0
68.0	27.5	51.4	—	—	—	—
139.0	10.0	18.7	17,160	22.6	57,380	75.4
192.0	5.0	9.3	9,200	12.1	67,020	88.0

* Eggs inseminated 20.5 minutes before initial measurment.
† Eggs inseminated 63 minutes before initial measurement.

In Experiment 7 (Table II, Fig. 1) at 63 minutes after insemination 3.5 ml. of *S. franciscanus* eggs were suspended in a liter of sea water containing 53.5 μg P/liter. The results are similar to those obtained in Experiment 6. The eggs remove orthophosphate from the external medium at a constant rate until the concentration falls below 20 μg P/liter, when the rate of uptake by the eggs falls off sharply (Fig. 1).

In Experiment 8 (Table III) at 43 minutes after insemination 4.9 ml. of *S. franciscanus* eggs were suspended in a liter of sea water. The P and P^{32} concentrations in the suspension fluid were measured at the beginning and at the end of successive 30-minute periods. Additional amounts of P and P^{32} were added to replenish the external medium prior to each 30-minute period. As in the two

previous experiments, the decrease in concentration of P^{32} parallels the decrease in concentration of P in the suspension fluid (Table III, columns 6 and 7). The results show that as long as the concentration of orthophosphate is over 18 to 20 μg P/liter, the rate of uptake of P and P^{32} remains fairly constant during the first 430 minutes after insemination (Table III, column 8).

Distribution of P^{32} between the trichloroacetic acid-soluble and -insoluble fractions of the eggs

Suspensions of *Lytechinus pictus* eggs were prepared containing one ml. eggs/liter of sea water maintained at a temperature of 20 to 21° C. Two hours after

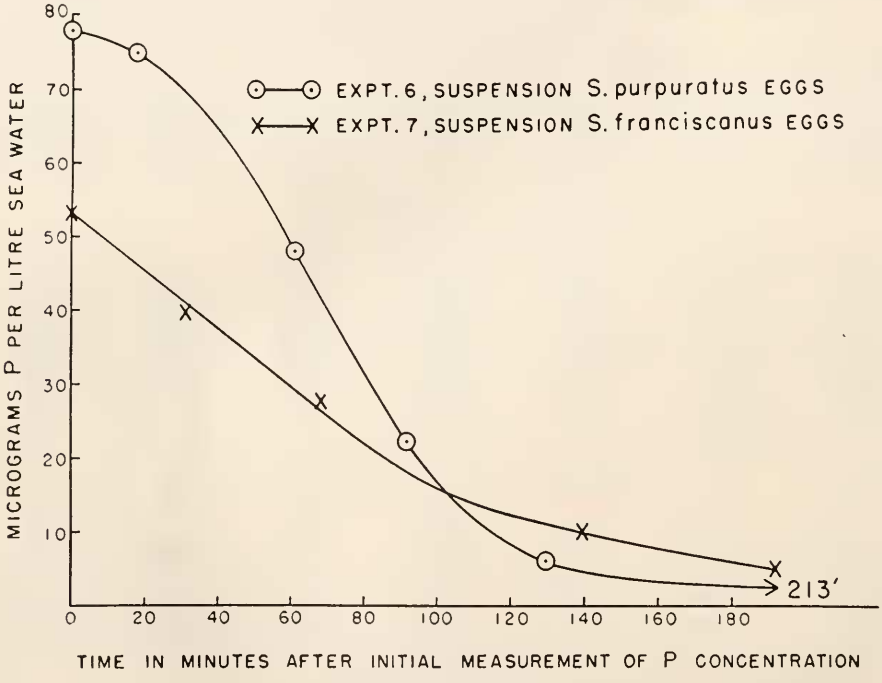

Figure 1.

the eggs had been removed from the ovaries, carrier-free P^{32} was added. At various intervals of time duplicate 20-ml. samples of the suspension were removed, the eggs washed three times by centrifugation at $86 \times g$ for three minutes in non-radioactive sea water, the supernatant sea water decanted, an equal volume of ice cold 10 per cent trichloroacetic acid added to each of the duplicate samples, the trichloroacetic acid-soluble and -insoluble fractions separated, and the P^{32} content of the fractions measured.

In unfertilized eggs, between 95.7 to 95.9 per cent of the P^{32} was recovered in the trichloroacetic acid-soluble extracts, with 4.1 to 4.3 per cent in the acid-insoluble fractions, after the eggs had been exposed to P^{32} for two hours.

In the process of washing the unfertilized eggs in non-radioactive sea water

prior to homogenization, from 4 to 8 per cent of the P^{32} initially present in the eggs was removed. The effect of this loss of P^{32} is to decrease the relative proportion of P^{32} contained in the trichloroacetic acid-soluble extracts of the washed eggs by 0.2 to 0.3 per cent.

In experiments carried out using fertilized eggs, the eggs were inseminated two hours after removal from the ovaries and P^{32} was added at the time of insemination. In eggs exposed to P^{32} for a period of 45 to 120 minutes after insemination, and washed for a period of 20 to 30 minutes in non-radioactive sea water, 96.3 to 96.4 per cent of the P^{32} was found in the trichloroacetic acid-soluble extracts, and 3.6 to 3.7 per cent in the acid-insoluble residue. No appreciable quantity of P^{32} is lost from fertilized eggs when washed in non-radioactive sea water (Brooks and Chambers, 1954). The slightly lower proportion of P^{32} in the acid-insoluble fraction of fertilized eggs, as compared to the unfertilized, may be entirely due to the loss of P^{32} from the unfertilized eggs when they are washed prior to homogenization.

TABLE III

Suspension of fertilized S. franciscanus eggs. Disappearance of P and P^{32} from the suspension fluid during thirty-minute periods, following successive additions of P and P^{32}. Experiment 8

Time after insem., in 30 minute intervals	Initial concentration:		Final concentration:		Per cent decrease P conc.	Per cent decrease P^{32} conc.	μg P/ml. eggs/min.
	μg P/l.	CPM P^{32}/l.	μg P/l.	CPM P^{32}/l.			
50 to 80	63.5	598,000	41.0	356,000	35	37	0.15
110 to 140	50.5	473,000	25.7	248,000	49	48	0.17
160 to 190	47.0	—	23.5	—	50	—	0.16
210 to 240	57.0	578,000	35.8	350,000	37	39	0.14
290 to 320	38.5	385,000	18.0	214,000	53	45	0.14
325 to 355	(16.0)	(220,000)	(10.0)	(140,200)	(37)	(35)	(0.04)
400 to 430	57.5	550,000	30.7	286,000	47	48	0.18

In a series of experiments, after exposing the inseminated eggs to P^{32} for 50 minutes and washing, the eggs were allowed to develop 400 minutes in non-radioactive sea water to the early blastula stage prior to homogenization. No P^{32} was lost from the fertilized eggs during the long period of development in the sea water free of P^{32}, even though the medium surrounding the eggs was repeatedly replaced by fresh sea water. The quantity of P^{32} found in the acid-soluble extract amounted to 93.0 per cent of the total, with 7.0 per cent in the acid-insoluble fraction, as compared to 96.4 and 3.6 per cent, respectively, in the corresponding experiment on fertilized eggs homogenized immediately after washing. The experiment reveals that a substantial portion of the phosphate, initially accumulated in the eggs, becomes incorporated in the acid-insoluble fraction. This conclusion is based on the consistency with which a lower percentage of P^{32} was found in the acid-insoluble residue of fertilized eggs continuously exposed to P^{32}.

In the control experiment P^{32} was added only after the eggs had been suspended in trichloroacetic acid. Even after repeated washing of the acid-insoluble residue with trichloroacetic acid, 1.0 per cent of the P^{32} was retained in this fraction. This experiment indicates that the quantity of P^{32} organically combined in the acid-

insoluble residue is probably less by at least one per cent than the quantities actually found.

The distribution of P^{32} between the trichloroacetic acid-soluble and -insoluble fractions of *S. purpuratus* eggs, both unfertilized (four experiments) and fertilized (six experiments) is essentially identical to that found in the eggs of *Lytechinus pictus*.

Lipids and phospholipids were extracted from the acid-insoluble residue of the fertilized *L. pictus* eggs. The acid-insoluble residue, after complete extraction with a mixture of three volumes ethanol and one volume ether, retained 92.1 per cent of the original P^{32} content. The ethanol-ether extract was dried, and the residue extracted with petroleum ether. The petroleum ether fraction containing the phospholipids accounted for 7.5 per cent of the total P^{32} content of the trichloroacetic acid-insoluble fraction. The remaining 0.5 per cent was in the petroleum ether-insoluble fraction.

TABLE IV

Distribution of P and P^{32} in the acid-soluble extracts of Strongylocentrotus purpuratus eggs. Experiments 9, 10 and 11

Expt. No.	Condition of eggs	μg P/ml. eggs ± std. dev.			Per cent total P^{32}:		
		Inorg. P	Labile P	Inorg. + labile P	Inorg. P^{32}	Labile P^{32}	Acid stable P^{32}
9	Unfertilized	58±.6	408±4	466±4	24	66	10
	Fertilized	16±.2	456±5	472±5	6	88	6
10	Unfertilized	69±.7	415±4	484±5	—	—	—
	Fertilized	34±.4	451±5	485±5	8	84	8
11	Unfertilized	77±.8	460±5	537±5	—	—	—
	Fertilized	39±.4	496±5	535±5	9	84	7

Distribution of P and P^{32} in the trichloroacetic acid-soluble extracts of S. purpuratus eggs

The quantities of inorganic P and P liberated after 10 minutes' hydrolysis in 1 N HCl at 100° C. in the trichloroacetic acid-soluble extracts of unfertilized and fertilized eggs were determined. The results of three representative experiments are shown in Table IV, Experiments 9, 10 and 11. Five ml. of *S. purpuratus* eggs were suspended in a liter of sea water containing 20 to 50 μg P as orthophosphate/liter. The suspension was divided into two equal lots. Carrier-free P^{32}, 1 μc/100 ml. suspension, was added to one lot of unfertilized eggs one hour after removal from the ovaries. The other lot was inseminated two hours after the eggs had been removed from the ovaries, and at the same time duplicate 100-ml. samples were removed from the suspension of unfertilized eggs, centrifuged, and the trichloroacetic acid extracts prepared. Thirty minutes after insemination 0.1 μc P^{32}/100 ml. suspension was added to the fertilized eggs, and at 60 minutes after insemination, duplicate 100-ml. samples were removed, the fertilized eggs washed twice in non-radioactive sea water by centrifugation, and the trichloroacetic acid extracts pre-

pared. The results show that following insemination, a prominent decrease in the concentration of inorganic P occurs within the eggs (Table IV, column 3), and at the same time a corresponding increase in the concentration of P liberated after hydrolysis (Table IV, column 4). Within the errors of the measurements, the sum of the inorganic P and P liberated after hydrolysis is the same both before and after insemination (Table IV, column 5).

The distribution of P^{32} between the various P fractions in the trichloroacetic acid extracts is shown in Table IV, columns 6, 7 and 8. The results reveal that the major portion of the P^{32} is associated with easily hydrolyzable organic P compounds. Following insemination, with the accompanying decrease in quantity of inorganic P and the increase in amount of P liberated after hydrolysis, the proportion of P^{32} in the easily hydrolyzable P fraction increases markedly. The proportion of P^{32} in the acid-stable P compounds is small, in spite of the fact that Whiteley (1949) reports the presence, in trichloroacetic acid extracts, of 511 μg acid-stable P/ml. *S. purpuratus* eggs, which amounts to approximately one-half of the total P content in the acid-soluble extract.

Discussion

The experiments presented in this paper establish conclusively that the entry of P^{32} into the fertilized eggs quantitatively measures the accumulation of orthophosphate within the eggs. However, P^{32} probably enters unfertilized eggs by an exchange process, since the quantity of orthophosphate in the medium surrounding the eggs either remains constant, or slowly increases. Measurements of the distribution of P^{32} in the trichloroacetic acid-soluble extracts of the eggs reveal that in both unfertilized and fertilized eggs the P^{32} is confined primarily to the intracellular inorganic phosphate fraction and the easily hydrolyzable organic P compounds. In fertilized eggs, between 84 to 88 per cent of the P^{32} entering the eggs is found in the easily hydrolyzable fraction, indicating that the accumulation of phosphate by fertilized eggs involves primarily its incorporation in the easily hydrolyzable P compounds.

In cells actively metabolizing substrate the intracellular inorganic phosphate concentration may be markedly lower than in slowly metabolizing cells, devoid of or with a limited supply of substrate (yeast: MacFarlane, 1936, 1939; bacteria: Wiggert and Werkman, 1938, O'Kane and Umbreit, 1942; brain tissue: Schachner *et al.*, 1942; retinal tissue: Bumm and Fehrenbach, 1931; liver: Lundsgaard, 1938). Furthermore, many investigators have shown that orthophosphate rapidly enters and accumulates in actively metabolizing cells (diatoms: Ketchum, 1939a, 1939b; yeast: Hevesy *et al.*, 1937, Mullins, 1942; bacteria: Vogler and Umbreit, 1942, Wiggert and Werkman, 1938, O'Kane and Umbreit, 1942, Hotchkiss, 1946; brain tissues: Schachner *et al.*, 1942). When the same cells are devoid of substrate, phosphate ions enter slowly and the cells may even lose phosphate to the external medium.

Unfertilized sea urchin eggs, at least after a period of sojourn in sea water, present the picture of cells with limited available or utilizable substrate. They possess a characteristically low metabolic rate (Borei, 1948), have a high inorganic phosphate content, a relatively low content of easily hydrolyzable P (see also

Chambers and Mende, 1953b), may slowly lose phosphate to the external medium, and P penetrates the eggs at an extremely slow rate (Brooks and Chambers, 1954). However, after the eggs are fertilized, the eggs behave as if an abundant supply of substrate had been made available, or had become utilizable. The oxygen consumption increases, the inorganic phosphate content of the eggs is strikingly lowered, the quantity of easily hydrolyzable P increases (see also Chambers and Mende, 1953b), and the eggs now accumulate phosphate, absorbing it from the external medium.

The fertilized eggs would appear to accumulate orthophosphate against a concentration gradient of a thousand-fold or more (compare column 2, Tables II and III with column 3, Table IV). This, however, is unlikely since the analytically determined inorganic P content of cells probably represents, in addition to the true intracellular ionic orthophosphate, hydrolysis products of highly labile phosphate esters and orthophosphate which, in the living cell, had been present in undissociated salt-like complexes. The binding of orthophosphate by electrostatic forces has been shown to occur, for example, in the protein aldolase (Velick, 1949). Denaturation of proteins may abolish their ability to bind anions (Klotz and Urquhart, 1949). Furthermore, the anions of an extracting agent, such as trichloroacetic acid, would tend to displace phosphate ions which, in the living cells, had been present in undissociated salt-like complexes and in equilibrium with free orthophosphate ions. It is proposed that in the living sea urchin eggs the actual concentration of free ionic orthophosphate is only a fraction of the analytically determined inorganic P. Following fertilization of the eggs, along with the demonstrated decrease in concentration of the analytically determined inorganic P, the concentration of free orthophosphate ions in the egg protoplasm may be reduced to such a low order of magnitude as to favor the entry of orthophosphate from the external medium.

The hypothesis has been advanced that the penetration of orthophosphate into cells requires esterification at the cell surface. The marked effects of changes in temperature and of certain metabolic inhibitors (Kamen and Spiegelman, 1948, Villee *et al.*, 1949) on the rate of penetration of orthophosphate have been cited in support of this hypothesis. However, Jacobs and co-workers (1935) have emphasized that changes in temperature may cause marked shifts in "equilibrium" states, and such alterations would have to be taken into account before the effects of temperature changes on the rate of penetration of orthophosphate could be properly evaluated. Similarly, metabolic inhibitors must induce profound changes in "equilibrium" states within cells. For example, Spiegelman, Kamen and Sussman (1948) have shown that azide prevents the decrease in concentration of intracellular inorganic P which normally occurs when yeast ferments glucose.

The claim has also been made that orthophosphate must enter cells by a process of esterification at the cell surface, since the specific activity of the P in certain organic compounds may be higher than that of the intracellular inorganic P (*e.g.*, Lindberg, 1950). Such an interpretation from specific activity measurements is open to serious question, since the analytically determined inorganic P of cells is undoubtedly derived from several different components, and does not represent the true ionic orthophosphate content of the living cell.

The observed great differences in the rates of penetration of orthophosphate into cells at different levels of metabolic activity may just as well be explained by

changes in "driving forces" such as the rate at which orthophosphate is esterified within the cells, and changes in the concentration gradient of free orthophosphate ions.

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Summary

1. The concentration of phosphate in the external medium of a suspension of unfertilized *Strongylocentrotus* eggs remains constant, or increases, while in a suspension of fertilized eggs, the concentration of phosphate in the external medium decreases.

2. Fertilized *Strongylocentrotus* eggs absorb P^{32} and phosphate from sea water at identical rates, revealing that the exchange of phosphate between the cell interior and the external medium is inappreciable.

3. The rate at which phosphate is removed from sea water by fertilized *Strongylocentrotus* eggs is relatively independent of the external concentration as long as this exceeds 15 to 20 micrograms P per liter.

4. When unfertilized and fertilized sea urchin eggs are continuously exposed to sea water containing P^{32} and more than 20 micrograms P per liter, 95.9 to 96.4 per cent of the P^{32} which enters the eggs is found in the trichloroacetic acid-soluble fraction, with 3.6 to 4.1 per cent of the P^{32} being recovered in the acid-insoluble fraction. The distribution of P^{32} between these two fractions is not significantly different in the unfertilized, as compared to the fertilized eggs. Although a slightly lower proportion of P^{32} was found in the acid-insoluble residue of unfertilized eggs, outward leaching of P^{32} during the washing of the unfertilized eggs may well account for the difference noted.

5. If fertilized *Lytechinus pictus* eggs containing P^{32} are suspended in a non-radioactive medium shortly after insemination, the proportion of P^{32} in the acid-insoluble fraction increases from 3.6 per cent at the two-celled stage to 7.0 per cent at the blastula stage.

6. The concentration of inorganic P in the trichloroacetic acid-soluble extracts of the eggs decreases prominently following insemination. A corresponding increase occurs in the quantity of P liberated after 10 minutes' hydrolysis of the extracts in 1 N HCl at 100° C.

7. The major portion of the P^{32} which enters the eggs is found in the easily hydrolyzable P fraction of the trichloroacetic acid-soluble extracts. After fertilization, the proportion of P^{32} in the easily hydrolyzable P fraction increases.

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